(19)(CA) **CANADIAN PATENT** (12)

(54) DNA Probe Diffraction Assay and Reagents

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FIELD OF THE INVENTION

This invention relates to an improved assay method and reagents therefor. In particular this invention relates to a light diffraction assay method using DNA sequences as probes, and
5   reagents which provide enhanced sensitivity.

BACKGROUND OF THE INVENTION

Many solid-phase immunoassays involve surface illumination and consequent light emissions from molecules attached to the surface.
10   Generally, these emissions travel in all directions. Either these divergent emissions must be collected with expensive and awkward light collection optics to achieve sensitivity or the inherent inefficiencies and consequent low signal to light level ratios must be accepted.

15   Diffraction gratings cause light to be diffracted into specific angles as contrasted to being scattered in all directions. The original gratings were prepared by ruling a number of straight, parallel grooves in a surface. Incident light is diffracted by each of the surfaces and is principally directed in directions in

20   which light from each groove interferes constructively with light scattered by the other grooves. This constructive light interference property of a grating allows efficient collection of light. Diffraction gratings have been used for dispersing light into its spectral components.

25   Many assay systems have been developed using different physically measurable properties of reagents to provide a measurement of an analyte concentration in a sample. Radioimmunoassay (RIA), immunofluorescence, chemiluminescence, enzyme immunoassays (EIA), free radical immunoassays (FRAT), light

30   scattering nephelometry, transistor bridge probes, indium reflective surfaces, and ultrasonic probes have been applied. These systems use the highly selective reaction between a primary binding reagent material such as an antibody or antigen and an

-2-

analyte selectively binding therewith. Due to limits of sensitivity, however, prior art systems have required the use of relatively large analytes, such as antibodies or large antigens, or require the use of sandwich assay techniques to increase the
5   detectable signal.

DESCRIPTION OF THE PRIOR ART
U.S. Patent 4,647,544 corresponding to European Patent Application 85304496.4 published January 8, 1986 describes a light
10   diffraction system and method, and biogratings therefor.

Disclosed methods for forming the biogratings comprise forming a coating of an active antibody on a flat surface such as a glass or plastic surface. In one disclosed method, the selective
15   destruction of antibody molecules to form stripe-like regions is accomplished with an intense UV source such as a mercury lamp together with a shadow mask placed near or in contact with the coated surface. Binding of analytes with the stripes of active antibody yields a light-diffracting grating. In the sandwich assay
20   and competition assay applications of this technology, a labeled reagent ligand can be used to enhance the mass of a bound material.
Photoresists and laser light have been used to make holographic gratings. The coarseness of the photographic medium restricts the groove spacing. Use of photoresist masks in the production of
25   precisely configured shapes and lines in semiconductor material is widely used in the production of semiconductor devices. The masks can be placed on or very near the surface to be exposed as described in U.S. Patent 4,647,544. In this approach, the best line definition is obtained by pressing the mask against the
30   surface to be exposed with high pressure to minimize light reflection at the edges of the masking elements.
U.S. Patent 4,521,522, issued to Lundstrom, et al., presents a method for determining antigen-antibody binding using reflected electromagnetic radiation

-3-

The biogrids and biogratings of the prior art have been limited by the resolution of the systems used. The biogratings have generally been limited to assays involving determination of antibodies or relatively large antigens, or the use of sandwich
5   assays.

The analysis and detection of minute quantities of substances in biological samples has become a routine practice in clinical and analytical laboratories. DNA probes are used in a technique based on nucleic acid hybridization (polynucleotide sequence-based
10   techniques). These are characterized by a sequence of steps comprising the non-covalent hybridizing of a labeled polynucleotide sequence or probe to a complementary sequence of the analyte under hybridization conditions. Such procedures are described by M. Grunstein, et al., *PNAS, USA* 72:3961-3965 (1975).
15   DNA hybridizations have taken place *in situ*, i.e., within tissue samples, or *in vitro*, i.e., with substances which have been separated from tissue. This invention relates to such *in vitro* DNA hybridizations, and improved detection thereof.

It is desirable to detect when hybridization takes place
20   between the reagent polynucleotide sequence and the complementary sequence of the analyte. This detection has been effected in the prior art by using labeled probe sequences. The test sample is bound to a support, and labeled probe is contacted thereto. Sufficient time is allowed for hybridization, and excess probe is
25   removed. The presence and amount of label present in the sample corresponds to the presence and amount of the analyte. Alternatively, the DNA probe may act as a primary binding agent in a sandwich assay, and a labeled second binding partner for the probe is used to amplify the signal. Common labels include
30   radiolabels, enzyme labeling, and immunofluorescent or chemiluminescent labels.

Exemplary DNA assays are described in U.S. Patent 4,563,417, which describes a nucleic acid hybridization assay using antibodies to intercalation complexes, and in U.S. Patent 4,689,295, which describes a DNA probe test for salmonella.

SUMMARY OF THE INVENTION

The improved assay of the subject invention uses DNA sequences as probes in a nucleic acid hybridization diffraction assay to detect specific DNA sequences in a sample. Diffraction assay

5   methodologies are applied to determine the presence and amount or analyte.

This invention involves a discovery in the area of supporting surfaces for the biogrid or biograting which provide greatly reduced non-specific hybridization or binding. A preferred process

10   of this invention involves manufacturing a biograting for use in a light diffraction assay, and comprises adhering a uniform layer of hybridizing reagent comprising a nucleotide sequence on a smooth, solid surface and exposing the surface to UV radiation through a shadow mask with diffraction grating pattern of lines to

15   selectively deactivate the hybridizing reagent, leaving a biological diffraction grating design of lines of active hybridizing reagent. The smooth, solid surface is a semiconductor, preferably selected from the group consisting of polysilicon and single crystalline silicon surfaces. By use of the process, the

20   sensitivity of the biogrid is improved such that a nucleotide sequence may be applied to a biogrid surface, and the biogrid used in a nucleic acid hybridization assay.

Another preferred biograting of this invention for use in a light diffraction assay comprises a surface preferably selected

25   from the group consisting of polysilicon and single crystalline silicon surfaces having on said surface, a biological diffraction grating design of lines of active hybridizing reagent which is the product of the process of adhering a uniform layer of a nucleic acid sequence on a smooth, solid surface and exposing the surface

30   to UV radiation through a shadow mask with diffraction grating pattern lines to selectively deactivate the hybridizing reagent nucleic acid sequence to leave a biological diffraction grating design of lines of active nucleic acid sequence, wherein a precise

focused shadow is cast on the nucleic acid sequence layer without physically contacting the hybridizing reagent layer with the shadow mask.

Because of the improved sensitivity obtained with this invention, no enhancement of signal output with a labeled secondary reagent is required.

The diffraction hybridizing assay method of this invention for determining the presence or quantity of an analyte in an aqueous sample comprises contacting a nucleic acid sequence diffraction biogrid with the sample under proper circumstances and for a sufficient time to permit nucleic acid hybridization between a nucleic acid sequence probe and an analyte; separating the biogrid from the sample; illuminating the biogrid with light from a light source, and determining the light diffracted by the diffraction hybridization assay surface.

The test sample to be assayed can be any medium of interest, and will usually be a liquid sample of medical, veterinary, environmental, nutritional, or industrial significance. Human and animal specimens and body fluids particularly can be assayed by the present method, including urine, blood (serum or plasma), milk, cerebrospinal fluid, sputum, fecal matter, etc. The analyte may pertain to normal, healthy states, or may represent a deviant or diseased state. Where the test sample to be tested contains principally double stranded nucleic acids, such as contained in cells, the sample will be treated to denature the nucleic acids, and if necessary first to release nucleic acids from cells. Denaturation of nucleic acids is preferably accomplished by heated in boiling water or alkali treatment, which, if desired, can simultaneously be used to lyse cells. Also, release of nucleic acids can, for example, be obtained by mechanical disruption (freeze/thaw, abrasion, sonication), physical/chemical disruption (detergents such as TWEEN,* TRITON,* SDS,* alkali treatment, osmotic shock, or heat), or enzymatic lysis (lysozyme, proteinase K,

*trade-mark

-6-

pepsin). The resulting medium will contain nucleic acids in single stranded form which can then be assayed according to the present hybridization method.

5   BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic of incident and diffracted light designs in the method of this invention.

Fig. 2 is a schematic view of a stationary platform embodiment of the diffraction assay apparatus of this invention.

10   Fig. 3 is a schematic view of a rotating platform embodiment of the diffraction assay apparatus of this invention.

Fig. 4 is a cross-sectional view of a dipstick having mounted thereon, a plurality of insoluble supports with diffraction grating designs of nucleic acid sequence reagents on the surfaces thereof.

15   Fig. 5 is a fragmentary, magnified cross-sectional view of an insoluble support having nucleic acid sequence hybridizing reagent on the surface thereon in a diffraction grating design.

Fig. 6 is a schematic representation of the process for manufacturing an insoluble support with the diffraction grating
20   design of Fig. 4.

                DETAILED DESCRIPTION OF THE INVENTION

This invention is an improvement of an assay applying light diffraction. By forming a grating of hybridized reagent-analyte
25   complexes on the surface of an insoluble support, the incident light can be diffracted into a discrete series of angles, and the light can be detected and measured with a high efficiency. The angle of diffraction is a function of the grating line spacing and the wavelength of the incident light.

30   The term "analyte" is used herein to designate a substance or substances, either alone or in admixtures, whose presence is to be detected, and, if desired, quantified. The analyte may be a DNA or RNA molecule of small or high molecular weight, a molecular complex

-7-

including those molecules, or a biological system containing nucleic acids, such as a virus, a cell, or a group of cells. Among the common analytes are nucleic acids, (DNA or RNA) or segments thereof, either single- or double-stranded, viruses, bacteria,

5 cells in culture and the like. Bacteria, either whole or fragments thereof, including both gram positive and gram negative bacteria, fungi, algae and other microorganisms such as Mycoplasmatales, spores, parasites, or yeast are also analytes, as well as animal (e.g., mammalian) and plant cells and tissues. The analyte sample

10 is treated to obtain single-stranded nucleotide sequences which are hybridizable to the nucleic acid sequence of the probe.

The term "probe" refers to nucleotide sequence which is complementary to a nucleotide sequence of a particular analyte and which hybridizes to said analyte nucleotide sequence. The probe

15 may be derived from single or double stranded DNA, RNA, either as a nucleic acid sequence or as nucleic acid sequence contained within a suitable plasmid. Alternatively, the nucleic acid probe may be synthesized by conventional procedures well known in the art. The nucleic acid sequence may be monoclonal or polyclonal, and may be

20 of any appropriate length. Preferred lengths are from 0.1 to 10 Kb, more preferably from 0.2 to 3.0 Kb, and especially from 0.5 to 1.0 Kb. For hybridization, it is necessary to ensure that the nucleic acid is single-stranded. For double-stranded DNA probes, this is usually achieved by boiling or by denaturing in alkali.

25 The probe is bound to an insoluble support and acts as the primary hybridization reagent.

The term "hybridization assay" or "binding assay" is used herein to designate an assay using any hybridizing reaction between a DNA probe and an analyte which is hybridizable therewith.

30 The term "light disturbing", as used herein, is defined to include all ways in which light is affected including light absorbing, reflecting, scattering, refracting and phase changing.

The term "diffraction grating design", as used herein, is defined to include a pattern of stripes or lines consisting of active hybridizing agent alternated with inactivated or denatured hybridizing reagent. The active hybridizing agent and inactivated

5   hybridizing agent preferably possess very similar light scattering efficiencies so that the stripes or lines of the diffraction grating design are essentially uniform to light diffraction in the absence of analyte, that is, light is scattered equally strongly from each type of strip before analyte binds preferentially to the

10   active hybridizing agent stripes or lines. The diffraction grating design becomes a diffraction grating with the hybridizing of a light-disturbing analyte.

A "biogrid" or "biograting" refers to a diffraction grating design integrated with a suitable supporting material.

15   The term "diffraction grating", as used herein, is defined to include reflection amplitude gratings which are formed in one or more steps. In one step gratings, the diffraction grating is formed directly by the binding of the non-light disturbing reagent on the insoluble surface with an analyte to yield a light

20   disturbing grating pattern. In multistep gratings, the binding product of the reagent on the insoluble surface with the analyte is non-light disturbing, and a second binding with a second binding reagent which is preferably labeled with a moiety which increases the light disturbance. Types of single and multistep gratings

25   formed in the process of this invention include reflection amplitude gratings, transmission amplitude gratings, reflection phase gratings, and transmission phase gratings. In reflection amplitude gratings in one or more steps, light is reflected from the grating, and the amplitude of the reflected light is modulated

30   by the spatially variable reflection of the grating. In transmission amplitude gratings in one or more steps, light is transmitted through the grating, and the amplitude of the transmitted light is modulated by the spatially variable

-9-

transmission of the grating. In the reflection phase grating in one or more steps, the light is reflected from the grating, and the phase of the reflected light is modulated by the spatially variable refractive index of the grating. In the transmission phase gratings in one or more steps, light is transmitted through the grating, and the phase of the transmitted light is modulated by the spatially variable refractive index of the grating. In the method of this invention, the diffraction grating may function as one or more of these types of gratings concurrently, and all of these grating types are included within the diffraction gratings of this invention.

Fig. 1 is a schematic of incident and diffracted light designs produced by incident light impinging on the diffraction grating in the method of this invention. The incident light 2 impinges on a diffraction grating 4 on the surface of the insoluble support 6. Reflected light 8 (m = 0) is reflected at angle B^0 which is equal to angle A, the angle of incidence, measured with respect to the normal 10 to the grating surface 4. Diffracted light is diffracted by the grating 4 in a series of angles. The relationship between the angle of incidence and the angle of diffraction is provided by the basic grating equation:

$$m\lambda = d(\sin A - \sin B)$$

wherein m is the spectral order of wavelength λ, d the groove spacing, and A and B are the angles of incidence and diffraction, respectively, with respect to the normal to the grating surface. The first order diffractions 12 (m = -1) and 14 (m = 1) have angles B^{-1} and B^{+1}, respectively. The second order diffractions 16 (m = -2) and 18 (m = 2) have angles B^{-2} and B^{+2}, respectively, with respect to normal 10. Light is also diffracted at higher orders. The first and second order diffractions are shown by way of example, not as a limitation, and all diffractions having a significant intensity can be used in the method of this invention.

Fig. 2 is a schematic view of a stationary platform embodiment of the diffraction assay apparatus of this invention. The light

beam 19 from the light source 20 passes through chopper 22 and beam
splitter 24. The light passing the splitter passes through
collimator 26 and impinges on the diffraction grating 28 mounted on
the stationary support 29.

5   A diffracted beam leaving the grating surface 28 is represented
by single line 32 having an angle B with respect to the normal 30.
The reflected beam is not shown. The light is collected with lens
34, is passed through an aperture 36 and impinges on the light
sensor 38, generating an electrical signal which is carried to the

10   conventional lock-in amplifier and recorder system 40 by cable 42.
A split beam 43 of light diverted by the splitter 24 from the
primary beam 19 is directed against another light detector 44. The
electrical signal from the light detector 44 is fed by cable 46 to
the lock-in amplifier 40 as a reference signal for compensating

15   slow drift errors originating from the light source. Cable 48
provides a reference signal from the chopper 22 to the lock-in
amplifier 40 for the lock-in function. This function synchronizes
the chopper speed (the light bundles passing the chopper) with the
operation (opening) of the amplifier filter, thus reducing ambient

20   light noise. The components of the system and their individual
functions including the light source, splitter, chopper,
collimator, lens, light detectors and lock-in amplifier are
conventional and well-known in the art.

       The diffraction measuring apparatus schematically represented

25   in Fig. 2 can be constructed to change the angles A and B. The
light source 20, chopper 22, beam splitter 24 and collimator 26 can
be mounted on an arm which is mounted to rotate in a vertical plane
around the diffraction grating 28 to a preferred angle A. Angle A
is preferably within the range of from 10 to 80° and optimally

30   within the range of from 20 to 70°. Even more importantly, the
lens 34, aperture 36 and light detector 38 can be mounted on an arm
for rotation in a vertical plane around the diffraction grating 28
through angles B which place the lens and aperture in the paths of

the reflected light beam 8 and the diffracted light beams
represented by 12, 14, 16 and 18 in Fig. 1. In this manner, a
single detector system can be rotated to detect and measure the
reflected light and all desired orders of the diffracted light.

5 Fig. 3 is a schematic view of a rotating platform embodiment of
the diffraction immunoassay apparatus of this invention. The light
beam 50 from the light source 52 passes through the beam splitter
54. The light passing the splitter passes through collimator 56
and impinges on the diffraction grating 58 mounted on the rotating
10 support 60.

The diffracted light beam 62 leaving the grating surface 58 has
an angle B with respect to the normal 64 to the grating surface.
The light is collected with lens 66, is passed through an aperture
68 a J impinges on the light sensor 70, generating an electrical
15 signal which is carried to the conventional lock-in amplifier and
recorder system 72 by cable 74. A split beam 76 of light diverted
by the splitter 54 from the primary beam 50 is directed against
another light detector 78. The electrical signal from the light
detector 78 is fed by cable 80 to the lock-in amplifier 72 as a
20 reference signal for compensating slow drift errors originating
from the light source 52. Cable 81 provides a signal from the
platform rotating system 82 to the lock-in amplifier 72 for the
lock-in function. This function synchronizes the platform speed
(the periodic alignment of the grating and light diffraction
25 designs therefrom with the lens 66 and aperture 68) with the
operation (opening) of the amplifier filter, thus reducing ambient
light noise. The components of the system and their individual
functions including the light source, splitter, light detectors and
lock-in amplifier are conventional and well-known in the art.

30 The diffraction measuring apparatus schematically represented
in Fig. 3 can also be constructed to change the angles A and B.
The light source 52, beam splitter 54 and collimator 56 can be
mounted on an arm which mounted to rotate in a vertical plane

around the diffraction grating 58 to a preferred angle A. Angle A is preferably within the range of from 10 to 80° and optimally within the range of from 20 to 70°. Even more importantly, the lens 66, aperture 68 and light detector 70 can be mounted on an arm

5   for rotation in a vertical place around the diffraction grating 58 through angles B which place the lens and aperture in the paths of the reflected light beam 8 and the diffracted light beams represented by 12, 14, 16 and 18 in Fig. 1. As in the embodiment shown in Fig. 2, a single detector system can be rotated to detect

10 and measure the reflected light and all desired orders of the diffracted light.

Alternatively, a stationary array of lens, aperture and detector sets can be positioned at preset angles to coincide with the paths of the reflected and diffracted light for both of the

15 systems of Fig. 2 and Fig. 3. For most simple embodiments of these systems, a stationary lens, aperture and light detector set can be positioned in the path of a first, second or third order diffraction beam. Other suitable combinations of stationary and movable arrays of detectors will be apparent to a person skilled in

20 the art, and all of these permutations and combinations are intended to be within this invention.

The light sources 20 (Fig. 2) and 52 (Fig. 3) are preferably narrow band light sources which can include filtered light from an incandescent light bulb or sunlight. The most preferred light is

25 intense narrow band light which is collimated and optimally polarized. The narrow band frequency can be within the range of from 200 to 1400 nm having a band width within the range of from 10 to 80 nm and is optimally within the range of from 400 to 800 nm having a band width of from 10 to 20 nm or lower. The output power

30 or output energy level of the light source can be 0.1 milliwatts and higher: generally, the higher the power level, the better the results.

-13-

The optimum light sources are monochromatic light sources such
as lasers. Optimum monochromatic light sources are lasers such as
helium-neon laser, diode laser, diode pumped solid state laser,
argon ion laser, helium-cadmium laser, YAG, harmonics of YAG, ruby,
5   excimer and tunable dye lasers. The light can be polarized or not
polarized. Linear polarization in the plane of incidence is
optimum.

    The light chopper 22 (Fig. 2) can be any conventional light
chopper. It can comprise a rotating plate defining evenly radially
10   spaced windows at a constant distance from the axis of rotation of
the plate.

    A beam expander can be optionally provided in the light paths
19 and 50 to increase the cross-sectional area of the beams and
increase the surface area of the diffraction grating interacting
15   with the light to increase sensitivity. Preferred beam expanders
are conventional lens or prism systems which convert an incident
collimated light beam into an emitted collimated beam of larger
size.

    The collecting lenses 34 (Fig. 2) and 66 (Fig. 3) collect each
20   of the diffracted light beams and concentrate or focus them into
smaller beams having a cross-sectional area less than the surface
area of the light detectors 38 and 70. Any conventional lens
system can be used which provides the desired reduction or
focusing.

25   The light detectors 38 and 70 can be any devices which can
quantitatively convert incident light intensity to voltage or
current in a proportional manner. Conventional light detectors
such as photomultiplier tubes or semiconductor-based detectors such
as silicon, germanium or gallium arsenide can be used.

30   The amplifier and recorder systems 40 and 72 can be any
conventional system which is customarily used for amplifying an
input signal from a light detector and recording a value
functionally related thereto. A simple system can comprise a bias

-14-

supply and an oscilloscope adjusted to show the amplitude of the output signal of each light detector 38 and 70. For more sensitivity, a lock-in amplifier which uses a reference signal from the chopper (Fig. 2) or rotating mount (Fig. 3) to perform the

5 functions described above. These amplifiers are standard, commercially available systems fully within the existing skill of the art and are not a part of this invention.

The platform 6 (Fig. 1) is designed to support a diffraction grating in a precise position of alignment with the incident light

10 beam 2. The configuration of the platform 6 is determined by the shape of the diffraction surface support and whether more than one diffraction surface is mounted on a diffraction surface support.

Fig. 4 is a cross-sectional view of a dipstick having mounted thereon, a plurality of insoluble supports with non-light

15 disturbing diffraction grating designs of reagents on the surfaces thereof. The dipstick body 130 has a plurality of insoluble support surfaces 134 having a diffraction grating design of hybridizing reagent coated thereon. The materials from which the dipstick is made should be nonbinding to minimize non-specific

20 binding during the assay procedure. Suitable dipstick surface materials include polyolefins such as polyethylene and polypropylene, hydrophilic polysilicon and polysiloxane polymers, and the like.

The support for the diffraction grating supports can be any

25 articles upon which the diffraction grating support surface can be mounted. The description of dipsticks are provided by way of example, and not as a limitation. Other articles such as microwells, plates, cavities and the like can be used. For many applications, dipsticks are a preferred embodiment.

30 Fig. 5 shows one embodiment of a diffraction grating element of this invention. Fig. 5 is a fragmentary, magnified cross-sectional view of an insoluble support having primary hybridizing reagent on the smooth surface thereof in a non-light disturbing diffraction

grating design. The insoluble support 140 of this embodiment has a smooth upper surface 142 upon which the diffraction grid design or pattern is coated. The diffraction grating design comprises a plurality of lines 144 of non-light disturbing active primary
5   hybridizing reagent thinly coated and adhering to the upper surface 142. The lines 144 are separated by lines 146 of non-light disturbing deactivated primary hybridizing reagent, for example, primary hybridizing reagent which has been deactivated by exposure to ultraviolet (UV) radiation, other deactivating radiation or
10   other deactivation energy.

The grating can have any number of lines 144 which will provide a diffraction design with the incident light wavelength in the presence of bound analyte. The preferred density of lines 144 are from 250 to 10,000 lines per cm for polarized monochromatic light
15   having a wavelength of from 600 to 800 nm. The optimum density of lines 144 is from 1250 to 2500 lines per cm for polarized monochromatic light having a wavelength of from 600 to 850 nm. The lines 144 have a preferred width (a) of from 0.5 to 20 microns and an optimum width of from 2 to 8 microns. The spacing "d" between
20   the center of adjacent lines 144 is preferably from 0.5 to 20 microns and optimally from 1 to 12 microns. The lines 144 and 146 can be of any desired thickness, depending upon the nature of the primary hybridizing reagent. For nucleic acid sequence coatings adhered to the surface 142 by adsorption or other bonding
25   procedures, the coating thickness is preferably a thin coating, yielding a layer which is non-light disturbing. Prior to the binding of the diffraction grating design with a light disturbing substance, the grating design is non-diffracting, that is, no diffraction occurs when it is exposed to light. When the active
30   primary hybridizing reagent 144 is bound with a light disturbing substance, the grating becomes a diffracting grating. The proportion of light diffracted is a function of the concentration or quantity of light disturbing materials bound with the coating

144, and this value can be used to verify the presence of analyte
and to quantify the concentration of analyte present in a sample.

The smooth upper surface 142 of the insoluble support can be
any material to which a primary hybridizing reagent can be adhered
5   by physical or chemical bonding and which will not interfere with
the reactions which are used to determine the presence and extent
of the hybridizing reaction. Organic and inorganic polymers, both
natural and synthetic, can be used as the insoluble support.
Examples of suitable polymers include polyethylene, polypropylene,
10   polybutylene, poly(4-methylbutylene), butyl rubber, silastic
polymers, polyesters, polyamides, cellulose and cellulose
derivatives (such as cellulose acetate, nitrocellulose and the
like), acrylates, methacrylates, vinyl polymers (such as polyvinyl
acetate, polyvinyl chloride, polyvinylidene chloride, polyvinyl
15   fluoride, and the like), polystyrene and styrene graft copolymers,
rayon, nylon, polyvinylbutyrate, polyformaldehyde, etc. Other
materials which can be used as the insoluble support can be silicon
wafers, glass, insoluble protein coatings on a smooth insoluble
surface, metals, metalloids, metal oxides, magnetic materials,
20   materials used in semiconductor devices, cermets and the like.

The preferred diagnostic supports of this invention have a
superior adsorptive quality for physical, non-covalent binding of
nucleic acid sequences, including primary hybridizing reagents and
proteinaceous materials applied to reduce the non-specific binding
25   of the ultimate coating. We have discovered that materials
previously used in the manufacture of semiconductor devices have
superior qualities in this respect. Suitable materials include
polished single crystalline silicon, aluminum, epitaxial silicon
coatings, silicon nitride coatings, silicon dioxide coatings, and
30   polysilicon coatings. The optimum surfaces have a polysilicon
coating. The most optimum supports 140 are single crystal silicon
wafers having a polysilicon coating on the outer polished surface
thereof.

-17-

The polysilicon surface is preferably a thin film of polysilicon deposited on a suitable surface, preferably a highly polished surface such as a single crystalline silicon wafer. Silicon's lattice structure provides a highly uniform surface with

5 superior properties as an immunoreagent support. Polysilicon films were developed in the semiconductor industry as valuable dielectric materials. They are used as gate electrodes in MOS devices, for high value resistors, diffusion sources to form shallow junctions, as conductors and to ensure ohmic contact to crystalline silicon.

10 The term "polysilicon" as used herein is synonymous with the term "polycrystalline silicon". These films are conventionally prepared by chemical vapor deposition techniques. These films and method for their preparation are described by A.C. Adams in "Dielectric and Polysilicon Film Deposition", VLSI TECHNOLOGY. (S.M.Sze ed.)

15 New York: McGraw-Hill, pp 93-129 (1983) and the citations therein.

The surface upon which the polysilicon is coated can be any material which is stable at polysilicon deposition temperatures. It can be prepared by pyrolyzing silane at 600 to 650°C in a

20 partial vacuum. Lower pyrolysis temperatures are suitable with more reactive silicon sources such as disilane.

The primary hybridizing reagent can be bound to the insoluble support by adsorption, ionic bonding, van der Waals adsorption, electrostatic bonding, or other non-covalent bonding, or it can be

25 bound to the insoluble support by covalent bonding. Procedures for non-covalent bonding are described in U.S. Patent No. 4,528,267. Procedures for covalently bonding antibodies and antigens to insoluble supports are described by Ichiro Chibata in IMMOBILIZED ENZYMES. Halsted Press: New York (1978) and A. Cuatrecasas,

30 J. Bio. Chem. 245:3059 (1970).

Fig. 6 is a schematic representation of a process for preparing the insoluble support with a diffraction grating design of primary

hybridizing reagent shown in Fig. 5. Any smooth surface having the requisite high binding affinity for primary hybridizing reagent can be used in this process. For purposes of clear explanation and not by way of limitation, the process is described for a semiconductor
5   wafer with a polished surface bearing a polysilicon coating. It should be understood that the same, equivalent, or similar procedures can be applied for preparing diffraction gratings designs with primary hybridizing reagents with other high binding smooth surfaces.

10   With the preferred insoluble supports of aluminum, silicon nitride, silicon dioxide, single crystalline silicon, and in particular the polysilicon surfaces, the primary hybridizing reagent can be applied by simple adsorption. In one procedure for non-covalent adhesion of primary hybridizing reagent to the surface
15   of an insoluble support, a primary hybridizing reagent such as an nucleic acid sequence derived from single or double stranded DNA or RNA, is applied to the surface of a support such as a polysilicon surface 150 in an aqueous buffer solution 152. The buffered primary hybridizing reagent solution is placed in a container with
20   the support bearing the polysilicon surface and incubated at room temperature until adsorption occurs, for example for from 0.5 to 18 hours and preferably from 1 to 3 hours, at temperatures of from 4 to 40°C and preferably from 20 to 26°C. The polysilicon surface is then rinsed with a buffered saline solution and dried.

25   The primary hybridizing reagent will comprise at least one single stranded base sequence substantially complementary to or homologous with the sequence to be detected. However, such base sequence need not be a single continuous polynucleotide segment, but can be comprised of two or more individual segments interrupted
30   by nonhomologous sequences. These nonhomologous sequences can be linear, or they can be self-complementary and form hairpin loops. In addition, the homologous region of the probe can be flanked at the 3'- or 5'-termini by nonhomologous sequences, such as those

comprising the DNA or RNA of a vector into which the homologous
sequence had been inserted for propagation. Such nonhomologous
regions are preferably minimized.

Linear or circular single stranded polynucleotides can be used
5   as the probe element, with major or minor portions being duplexed
with a complementary polynucleotide strand or strands, provided
that the critical homologous segment or segments are in single
stranded form and available for hybridization with sample DNA or
RNA. Particularly preferred will be linear or circular probes
10   wherein essentially only the homologous probe sequence is in single
stranded form.

The concentration of primary hybridizing reagent in the buffer
solution is selected to provide the desired reagent density on the
polysilicon surface. The primary hybridizing reagent solution can
15   contain from 0.02 to 100 micrograms/ml of the primary hybridizing
reagent and preferably contains from 10 to 50 micrograms/ml of the
primary hybridizing reagent in a buffered solution having a pH of
from 6.0 to 9.5 and preferably from 7.0 to 8.5.

Hybridizing reagent DNA probes are chosen to bind selectively
20   with the analyte to be measured in a sample. Many such sequences
have been isolated including DNA sequences for adenoviruses, HCMV,
various tumors, normal and aberrant genotypes (e.g., adult or fetal
chromosomes), cell products of recombinant DNA processes, bacteria,
yeast, algae, etc. In general, the hybridizing reagent is selected
25   to bind specifically or selectively with the analyte, the material
for which a sample is assayed.

The hybridizing reagent probes can be derived from natural
materials, or can be synthesized by conventional procedures well
known in the art and which are not a part of this invention. In
30   general, they can be synthesized by the triester method and the
phosphite method, each involving the solid phase method and the
liquid phase method. Suitable procedures are described in
Tetrahedron Letters, 1979:3635 (1979), *Nucleic Acids*

-20-

Research, 8:5473, 5491, 5507 (1980) and *Nucleic Acids Research Symposium Series*, 7:281 (1980).

The nucleotide probe can be made with a polyadenine, polycytosine, or adenine-cytosine copolymer tail which can be bound to a suitable linking group which facilitates binding of the hybridization reagent to the smooth surface 150.

In an alternate procedure, the linker group can be linked to the pyrimidine base of uridine triphosphate through an allylamine linker arm, and the product can be used as a substrate for probe synthesis using DNA and RNA polymerases in vitro, following a modified procedure of P. Langer, et al., *Biochemistry* 14:2447-2457 (1975). The 5-(3-amine)allyluridine and deoxyuridine 5'-triphosphates are prepared by reaction with the olefin in the presence of a palladium catalyst.

The surface 150 with the coating 156 of primary hybridizing reagent thereon is then rinsed with deionized water, and dried.

A mask is prepared by photographic methods conventional in semiconductor manufacturing. For example, a mask having a plurality of diffraction grating pattern lines having the desired line density and line widths can be prepared on a quartz glass or other UV-transparent plate through a photoresist process similar to photography. The dark lines of the mask correspond to active primary hybridizing reagent areas desired on the ultimate surface.

In Step C of Fig. 6, the shadow mask 158 is positioned in at a preselected distance from surface 150 to project a precise shadow of lines on the surface 150 having a coating 156 of primary hybridizing reagent thereon using conventional projection alignment techniques. The mercury-xenon illuminator 161 projects an intense, uniform collimated beam of UV light onto a photomask 158. The photomask 158 is mechanically held by a mask holder 159 at the desired distance from the surface 156 to produce non-contact projection of precision images onto the coated surface. The proximity gap 162 can range from 5 to 20 microns from the coated surface 150.

The projection alignment equipment and the projection methods involved in the operation thereof are well known in the art and do not form a part of this invention. The ORIEL™ photolithography illuminator and the accessories therefor (Oriel Corporation,
5   Stratford, CT.) is suitable for this purpose.

In this method, contact of the coating 156 with the mask 158 is entirely avoided, a critical feature which provides the superior diffraction grid of this invention. The surface is exposed to the precisely focused ultraviolet radiation until the hybridizing
10   capability of the portions of the primary hybridizing reagent exposed to the radiation are substantially reduced, or preferably eliminated. To manufacture a precision grating design, the radiation should form a sharp image on the coated surface.

The ultraviolet radiation exposure required to deactivate
15   coating exposed thereto depends upon the primary hybridizing reagent. For nucleic acid sequence reagents, exposure times of from 5 minutes to 60 minutes, and preferably from 10 to 30 minutes is sufficient with a ultraviolet radiation having a wavelength such as 260 nm and a power of from 8 to 20 milliwatts oer cm^2.

20   This treatment reduces or eliminates the hybridization properties of the nucleic acid sequence in lines 146, leaving active nucleic acid sequence reagent in a diffraction grating design as the lines 144 of Fig. 5.

The coated substrate containing areas having nucleic acid
25   sequence in a diffraction grating design is then cut into smaller area chips 160, each chip having a size sufficient to perform a nucleic acid sequence hybridization assay. These chips are then mounted on a suitable diagnostic support, such as the dipstick shown in Fig. 4, and are used in hybridization assays.

30   For most purposes, hybridization can be carried out either in aqueous solution or in the presence of formamide. Formamide is preferred if elevated temperatures will degrade the probe. RNA-DNA RNA-RNA, and DNA-DNA hybridizations can be carried out in

-22-

formamide, but DNA-DNA hybridizations, only, should be carried out
in aqueous solutions. A carrier DNA (denatured DNA, e.g., from
calf thymus or salmon sperm) is included in the hybridization
solution. Hybridization methods are well known to those skilled in
5   the art, and are detailed in, for example, Maniatis et al. (1982)
Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring
Harbor, NY.

After hybridization, washing is carried out to remove
10   unhybridized analyte and to dissociate unstable hybrids.

The improved biogrid products of this invention provide
improved sensitivity in assay methods using light diffraction. One
such method comprises a first step of contacting the insoluble
support with the biological diffraction grating design, or pattern
15   of hybridizing agent thereon, with the sample to be assayed for an
analyte which hybridizes specifically with the hybridizing agent.
The insoluble support is contacted with the sample for a time
sufficient to permit binding of the hybridizing agent and the
analyte. The sample is then removed from the insoluble support
20   with a suitable buffered rinse solution. This procedure leaves the
diffraction grating of hybridizing agent coupled with the analyte.

Light disturbing gratings are directly formed with most
analytes. The binding of the hybridizing agent and the analyte
converts the hybridizing agent design into a diffraction grating.
25   The nucleic acid sequence of DNA, RNA, or DNA/RNA hybrid, or cell
or tissue sample hybridizes and adheres to the insoluble support in
a diffraction grating pattern, and light illuminating the surface
is diffracted.

If the hybridizing agent-analyte binding product is non-light
30   disturbing, a second step comprises binding the insoluble support
with a secondary binding reagent which, in conjunction with the
hybridizing agent-analyte binding product, is light disturbing.
The secondary binding reagent may be labeled with one or more

-23-

substances which increase the bound mass, yielding a
light-disturbing grid pattern by absorption, reflection,
scattering, refraction or phase changing. Specific labels include
chromophores and other light absorbing materials, and reflective

5   and light transmitting beads and other light reflecting and
scattering materials. Alternatively, the hybridizing agent analyte
binding product or the secondary binding reagent can be coupled
with a secondary hybridizing reagent which is labeled or which can
be specifically bound with a binding partner therefor which is

10   labeled with a chromophore or light absorbing material. This
alternative coupling can occur during or subsequent to the
secondary binding reagent treatment in the second step. In yet
another embodiment, the secondary binding reagent is not itself
light-disturbing, but the hybridizing agent-analyte-secondary

15   binding reagent unit acts as a light-disturbing substance.
          The choice of secondary binding reagent is made depending upon
the type of analyte being assayed. In general, the secondary
binding reagent is a nucleic acid sequence coupled to a label. The
secondary binding reagent may also be an antibody of a selected

20   class which binds specifically with the analyte, or with the
double-stranded hybridization product produced by the hybridization
of the primary hybridization reagent and the analyte.
          Suitable chromophore labels are any light absorbing pigment or
dye which absorbs light of the wavelength produced by the light

25   source. Coupling of the hybridizing reagent-analyte binding
product with such a chromophore converts the light diffracting
design into a diffraction grating. Alternatively, the light
absorbing label can be another type of light disturbing particle or
reflective material such as colloidal gold or silver, or latex

30   microspheres.
          In the alternative method which has increased sensitivity using
the improved biogrids of this invention, the secondary binding
partner label binds specifically with a tertiary binding reagent

-24-

which is labeled with a suitable light disturbing material.
Examples of binding pairs for which either partner can be used as
the secondary binding partner label are avidin-biotin, IgG
antibody-Protein A, hapten-antihapten antibody, and the like. If
5 the secondary binding reagent is an IgG antibody, for example, it
can be unlabeled, since the Fc chain portion thereof is a binding
partner for Protein A or anti-IgG antibodies.

Following conversion of the biological diffraction design to a
diffraction grating, the insoluble support is rinsed with distilled
10 or deionized water. Then the strength of the light diffraction is
measured with a suitable light diffraction instrument such as
illustrated in Fig. 1. The relative strengths of the light
diffracted is a function of the amount of primary hybridizing
reagent-analyte conjugate comprising the grating. By repeating the
15 above procedure with a prepared series of solutions containing a
range of different known concentrations of analyte therein, a
standard curve functionally related to the strength of the
diffracted light is obtained. By comparing the reading obtained
with the sample containing the analyte with the curve cbtained with
20 solutions containing known concentrations of the analyte, the
concentration of analyte in the sample can be determined.
Comparing the strengths of the first, second, third, etc. order
diffractions with each other and with the strength of the reflected
light directly provides an indication of the degree of binding with
25 the grating hybridizing agent.

In a competition assay alternative method using the improved
biogrids of this invention, the insoluble support with the
biological diffraction grating design of hybridizing agent thereon
can be contacted with a mixture of the analyte sample and a reagent
30 analyte labeled with a label which is light disturbing. The amount
of reagent analyte hybridizing with the primary hybridizing agent
will be an inverse function of the amount of analyte in the
sample. The density of the diffraction grating formed will

therefor have a functional relationship with the concentration of analyte in the sample, and this difference can be detected in the strength of diffracted light.

This invention is further illustrated by the following specific but non-limiting examples. Unless otherwise specified, percents are provided as weight percents and temperature as degrees Centigrade. Examples which have been carried out in the laboratory are set forth in the past tense, and examples setting forth procedures which are constructively reduced to practice herein are set forth in the present tense.

EXAMPLE 1

Single-Stranded Probe Preparation

A pBR322 plasmid is treated with Hind III restriction enzyme, and extracted with a mixture of phenol and chloroform to yield the desired pBR322 double-stranded DNA fragment having a molecular weight of 2.0 Kb. The pBR322 fragment is dissolved in TRIS buffer (0.01 M, pH 8.0) containing 0.001 M EDTA, to a final concentration of 0.5 μg/ml. The solution is heated to boiling (100°C) for five minutes and quickly chilled in an ice bath to produce single-stranded pBR322 DNA.

EXAMPLE 2

DNA Probe Coated Wafer

A polysilicon wafer is immersed in the solution from Example 1, containing the single stranded pBR322 DNA. Coating is permitted to proceed inside a refrigerator overnight.

Salmon sperm DNA is dissolved in deionized water, and denatured by boiling at 100°C for five minutes, then quickly chilled in an ice bath, to yield a carrier DNA solution.

The polysilicon wafer is removed from the coating solution, and immersed in the carrier DNA solution for two hours. The wafer is then removed from the carrier solution, rinsed with deionized water, and dried.

EXAMPLE 3

Diffraction Grating Pattern

A mask having a series of squares corresponding in size and shape to the ultimate biograting product and with diffraction grating lines having a line spacing of ten micrometers, a line width of five micrometers, and a line density of 1000 lines/cm is placed five micrometers away from the single-stranded pBR322 DNA coated wafer produced by the process of Example 1, using a mask alignment fixture. The mask surface is exposed to ultraviolet light having a wavelength of 260 nm for thirty minutes.

The wafer is then cut into square chips having a diffraction pattern of lines of active single-stranded pBR322 DNA thereon, and mounted in a receptive cavity of a dipstick.

EXAMPLE 4

Diffraction Assay for pBR322 Through Hybridization

Double-stranded pBR322 DNA fragments are dissolved in a mixture of SSC *(3.0 M NaCl, 0.3 M trisodium citrate, pH 7.0), formamide (Sigma); Denhardt's solution (1.0% w/v each of polyvinylpyrrolidone-360, Ficoll 500*(Pharmacia Fine Chemicals, Inc.), and bovine serum albumin) 20% Sodium Dodecyl Sulfate, salmon sperm carrier DNA, and deionized water (20:50:10:2.5:2.5:10, v/v). The solution is boiled at 100°C for five minutes, and quickly chilled in an ice bath. The solution is applied to the dipstick product of Example 2, and incubated at room temperature for two hours. After incubation, the biograting on the dipstick is rinsed thoroughly with distilled water.

The intensity of the light diffracted by the grating is then measured using polarized monochromatic light having a wavelength of 632.8 nm from a helium-neon laser.

*trade-mark

-27-

EXAMPLE 5
Determination of Salmonella

A sample of Salmonella DNA probe is obtained following the
procedures of U.S. Patent 4,689,295.

5   The single-stranded probe is
immobilized on a silicon wafer according to the procedure of
Examples 2 and 3. Bacterial DNA is then introduced, and
hybridization is allowed to proceed for 2 to 3 hours. After
incubation, the biograting is rinsed thoroughly with distilled

10   water, and the intensity of the light diffracted by the grating is
then measured using polarized monochromatic light having a
wavelength of 632.8 nm from a helium-neon laser.

The procedure is repeated with a series of positive controls,
each containing a known amount of the salmonella DNA, to establish

15   a reference comparison with which the intensity of the diffracted
light obtained with the unknown sample is compared.

EXAMPLE 6
Preparation of HCMV Probe

20   The EcoRI restriction endonuclease fragment O from human
cytomegalovirus (HCMV) strain AD169 (Tamashire et al. J.Virol.
p.547-556 (1982), Chou and Merigan NewEng.J.Med. 308:921
(1983)) is cloned into the pBR322 derivative pACYC184 which is used
to transfect E. coli strain HB101 Rec A, as described by Tamashire

25   et al. After propagation and purification at the insert-bearing
pACYC184, the plasmid is digested with restriction endonuclease
EcoRI and the 6.8 Kb O fragment of HCMV is purified by preparative
electrophoresis in 0.8% agarose gels using standard procedures
(Maniatis et al. Molecular Cloning, Cold Spring Harbor

30   Laboratory, Cold Spring Harbor, NY. (1982)). The double-stranded O
fragment is dissolved in deionized water, and denatured by boiling
at 100°C for five minutes, then quickly chilled in an ice bath to
produce single-stranded DNA.

EXAMPLE 7

Detection of HCMV in Urine

A biograting is produced by the methods of Examples 2 and 3, replacing the DNA probe of Example 1 with the HCMV probe of

5   Example 6.

The clinical urine specimen to be tested is prepared in a manner similar to that described by Chou and Merigan (*NewEng.J.Med.* 308:921 (1982)). After clarification of the sample and concentration of the HCMV phage particles by

10   centrifugation, they are resuspended in a minimum volume of 0.5 M NaOH and allowed to stand for 15 minutes. After neutralization with a minimum volume of SSC, formamide, Denhardt's solution, 20% SDS, carrier DNA, and deionized water (20:50:10:2.5:2.5:10, v/v), the solution is applied to the dipstick product of Example 2, and

15   incubated at room temperature for two to 3 hours. After incubation, the biograting on the dipstick is rinsed thoroughly with distilled water.

The intensity of the light diffracted by the grating is then measured using polarized monochromatic light having a wavelength of

20   632.8 nm from a helium-neon laser.

The procedure is repeated with a series of positive controls, each containing a known amount of HCMV sample, to establish a reference comparison with which the intensity of the diffracted light obtained with the patient serum sample is compared.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1.   A biograting for use in a light diffraction assay
5   comprising a smooth surface having on said surface, a
biological diffraction grating design of lines of active
hybridizing reagent.

2.   The biograting of claim 1 wherein the hybridizing
10   reagent is a nucleotide sequence.

3.   The biograting of claim 1 wherein the smooth surface
is selected from the group consisting of a single
crystalline silicon, aluminum, epitaxial silicon, silicon
15   nitride, silicon dioxide and polysilicon.

4.   The biograting of claim 3 wherein the smooth surface
is selected from the group consisting of polysilicon and
single crystalline silicon.
20

5.   The biograting of claim 1 wherein the biological
diffraction grating design of lines of active hybridizing
reagent is substantially non-light disturbing.

25   6.   The biograting of claim 1 wherein the biological
diffraction grating design of lines becomes light-
disturbing in the presence of hybridized analyte.

7.   A process of manufacturing a biograting for use in a
30   light diffraction assay, comprising:
      (a)   adhering a uniform layer of hybridizing reagent
           on a smooth, solid surface; and
      (b)   exposing the surface to UV radiation through a
           shadow mask with diffraction grating lines to
35   selectively deactivate the hybridizing reagent

- 30 -

8. The process of Claim 7 wherein the hybridizing reagent is a nucleotide sequence.

9. The process of claim 7 wherein the smooth surface is selected from the group consisting of a single crystalline silicon, aluminum, epitaxial silicon, silicon nitride, silicon dioxide, and polysilicon.

10. The process of claim 9 wherein the smooth surface is selected from the group consisting of polysilicon and single crystalline silicon.

11. The process of claim 7 wherein the biological diffraction grating design of lines of active hybridizing reagent is substantially non-light disturbing.

12. The process of claim 7 wherein the biological diffraction grating design of lines becomes light-disturbing in the presence of hybridized analyte.

13. A diffraction hybridizing assay method for determining the presence or quantity of an analyte nucleotide sequence in an aqueous sample comprising:

    (a) contacting a biograting with the sample for a time sufficient to permit binding of hybridizing reagent and analyte, the biograting comprising a smooth surface having on said surface, a biological diffraction grating design of active hybridizing reagent that hybridizes selectively with the analyte, wherein the biological diffraction grating design of active hybridizng reagent is substantially non-light disturbing and becomes light distrubing in the presence of hybridized analyte; and

(b)   illuminating the biograting with light from a
light source, and determining the light
diffracted by the biograting.

14.   The diffraction hybridizing assay method of claim 13
wherein the analyte nucleotide sequence is derived from a
body tissue.

15.   The diffraction hybridizing assay method of claim 13
wherein the sample is a body fluid.

16.   The diffraction hybridizing assay method of claim 15
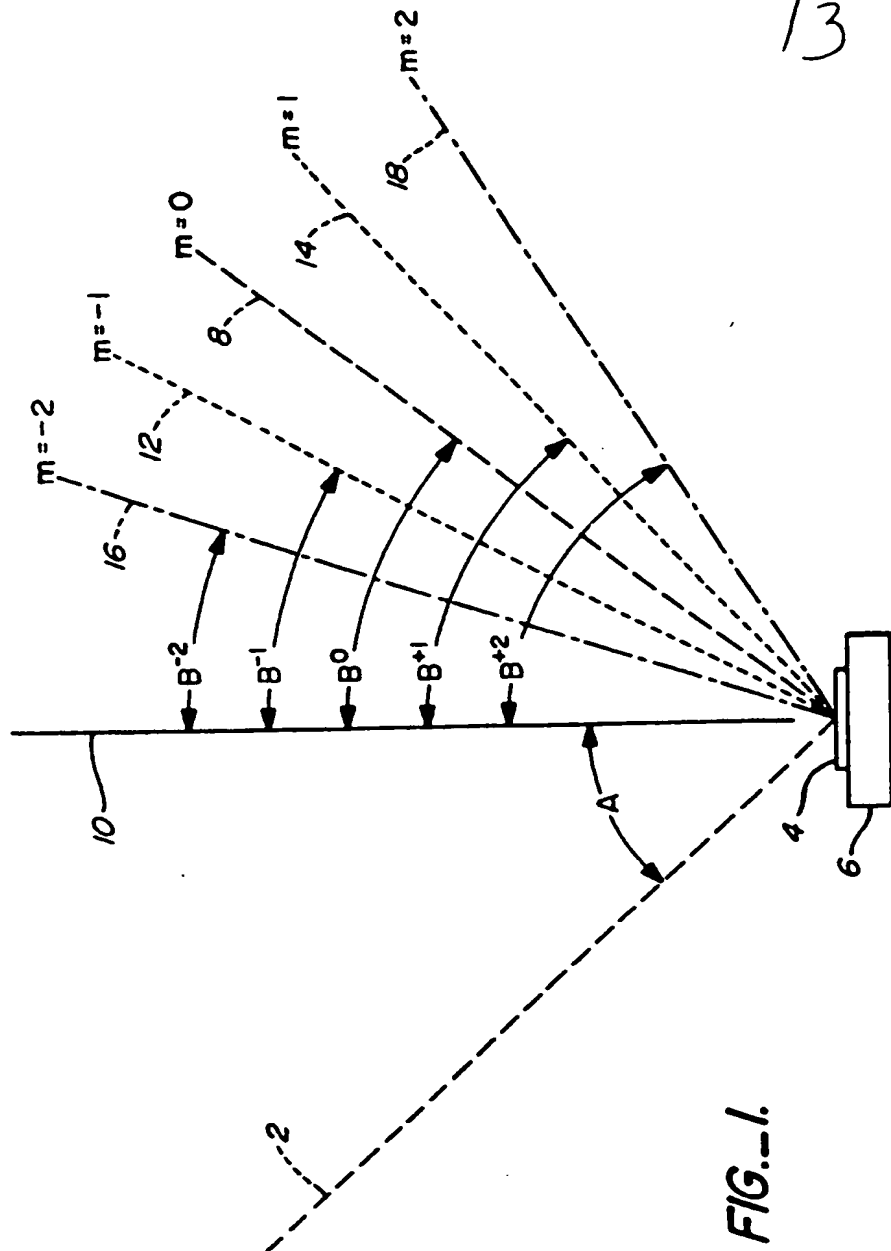wherein the sample is urine, blood serum, blood plasma,
milk, cerebrospinal fluid, mucus or sputum.

17.   The diffraction hybridizing assay method of claim 15
wherein the analyte nucleotide sequence is derived from
bacteria, viruses, fungi, algae, animal or plant cells.

18.   The diffraction hybridizing assay method of claim 13
wherein the analyte is a nucleotide sequence having origin
in a fetal cell, adult cell, bacterium, virus,
Mycoplasmatales, spore, algae, parasite, or yeast.

FIG._1.

FIG._2.

FIG._3.

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3/3

FIG._4.

130
134
146
144
146

FIG._5.

140
142
146
144
a
d
146
144

FIG._6.

150
A

152
150
B

161
159
158
162
156
150
C

160
D